Relation between the oligomerization state and the transport and phosphorylation function of the Escherichia coli mannitol transport protein: Interaction between mannitol-specific enzyme II monomers studied by complementation of inactive site- directed mutants

Boer, Harry; ten Hoeve-Duurkens, R.H.; Robillard, G.T.

Published in:
Biochemistry

DOI:
10.1021/bi9611016

Publication date:
1996

Relation between the Oligomerization State and the Transport and Phosphorylation Function of the *Escherichia coli* Mannitol Transport Protein: Interaction between Mannitol-Specific Enzyme II Monomers Studied by Complementation of Inactive Site-Directed Mutants†

Harry Boer, Ria H. ten Hoeve-Duurkens, and G. T. Robillard*

The Groningen Biomolecular Science and Biotechnology Institute (GBB) and the Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Received May 8, 1996; Revised Manuscript Received July 25, 1996⊗

ABSTRACT: Previous experiments with the mannitol-specific enzyme II of *Escherichia coli*, EII^{mtl}, have demonstrated that (1) the enzyme is a dimer, (2) the dimer is necessary for maximum activity, and (3) phosphoryl groups could be transferred between EII^{mtl} subunits [van Weeghel et al. (1991) *Biochemistry 30*, 1768−1773; Weng et al. (1992) *J. Biol. Chem. 267*, 19529−19535; Weng & Jacobson (1993) *Biochemistry 32*, 11211−11216; Stolz et al. (1993) *J. Biol. Chem. 268*, 27094−27099]. The experiments in this article address the mechanistic role of the dimer. They indicate that the A, B, and C domains of EII^{mtl} preferentially interact within the same subunit. Site-directed mutants in each of the three domains of EII^{mtl} were used to study phosphoryl group transfer by the EII^{mtl} dimer *in vitro* and mannitol transport *in vivo*. The C domain mutant, EII^{mtl}-G196D, which was unable to bind mannitol, and the separated C domain, IIC^{mtl}, which was unable to phosphorylate mannitol, formed a heterodimer which was capable of mannitol phosphorylation *in vitro* and mannitol transport *in vivo.* The rates of phosphorylation were approximately 10-fold lower in heterodimers containing two inactive subunits relative to the rates in heterodimers containing one inactive and one wild type subunit; phosphoryl group transfer through one subunit is kinetically preferred to intersubunit transfer. Heterodimers formed *in vivo* between one wild type EII^{mtl} subunit and the CB domain double mutant, EII^{mtl}-G196D/C384S, transported mannitol as rapidly as wild type EII^{mtl} alone; the presence of the inactive double mutant subunit did not reduce the transport rate. Thus, only one active A, B, and C domain in the dimer is sufficient for transport and phosphorylation activity, and if all three domains are situated on the same subunit, maximum rates are achieved.

Uptake and phosphorylation of the carbohydrate, mannitol, in *Escherichia coli* is catalyzed by the mannitol transport protein, enzyme II^{mtl}, which is located in the cytoplasmic membrane. EII^{mtl} [1] is a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) of *E. coli* (Lolkema & Robillard, 1992; Postma et al., 1993). Transport proteins belonging to this system are group translocation proteins that phosphorylate their carbohydrate substrates during transport. A series of phosphorylated proteins is involved in the phosphorylation of EII^{mtl}. Phosphoenolpyruvate (PEP) is the substrate of enzyme I which donates the phosphoryl group to a second protein, HPr. These two enzymes are general PTS proteins; all sugar-specific EII's are phosphorylated by P-HPr.

The mannitol transport protein consists of three domains, a membrane-bound C domain and two cytoplasmic domains, B and A (van Weeghel et al., 1991; Robillard et al., 1993). The A domain is phosphorylated at His554 by P-HPr. The phosphoryl group is transferred from His554 to Cys384 in the B domain, the second phosphorylation site on EII^{mtl} (Scheme 1) (Pas et al., 1988). The phosphoryl group is then transferred to mannitol which is translocated by the membrane-bound C domain (Grisafi et al., 1989; Lolkema et al., 1990).

Scheme 1

$$\text{P-HPr} + \text{EII}^{mtl} \rightarrow \text{HPr} + \text{EII}^{mtl}(\text{H554})\text{-P}$$

$$\text{EII}^{mtl}(\text{H554})\text{-P} \rightarrow \text{EII}^{mtl}(\text{C384})\text{-P}$$

$$\text{EII}^{mtl}(\text{C384})\text{-P} + \text{mtl (out)} \rightarrow \text{EII}^{mtl} + \text{mtl-P (in)}$$

The association state of EII^{mtl} has been the focus of attention with different techniques for many years. (i) Physical size measurements indicate that EII^{mtl} is an oligomeric protein with a mass that is most likely to be a dimer (Pas et al., 1987; Kandekhar et al., 1989; Lolkema et al., 1993; Boer et al., 1994). (ii) Dimeric forms of EII^{mtl} and

† This research was supported by the Netherlands Foundation for Chemical Research (SON), with financial aid from the Netherlands Organization for Scientific Research (NWO).

* To whom correspondence should be addressed. Telephone: 31-50-3634321. Fax: 31-50-3634165. E-mail: G.T.Robillard@chem.rug.nl.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; mtl, mannitol; mtl-P, mannitol 1-phosphate; man, mannose; glc, glucose; HPr, histidine-containing protein; EI, enzyme I of the phosphoenolpyruvate-dependent carbohydrate transport system; DTT, dithiothreitol; decylPEG, decylpolyethylene glycol 300; PEP, phosphoenolpyruvate. For the EII^{mtl} nomenclature, when referring to domains which are covalently attached, we use the terminology A domain, B domain, C domain, BA domain, etc. When referring to the domains which have been subcloned and expressed separately we use the nomenclature IIA^{mtl} for domain A of the mannitol-specific enzyme II, IIB^{mtl} for domain B of the mannitol-specific enzyme II, and IIC^{mtl} for domain C of the mannitol-specific enzyme II.

S0006-2960(96)01101-4 CCC: $12.00

the membrane-bound C domain were observed by gel electrophoresis after extraction of the protein out of the membrane (Roossien & Robillard, 1984a; Stephan & Jacobson, 1986). (iii) EII^{mtl} monomers could also be specifically cross-linked via sulfhydryl groups using either bifunctional sulfhydryl reagents or oxidants (Roossien & Robillard, 1984b; Roossien et al., 1986). (iv) Kinetic studies on the wild type enzyme indicated the existence of oligomeric forms of EII^{mtl} (Saier et al., 1980; Leonard & Saier, 1983; Roossien et al., 1984; Lolkema & Robillard, 1990), as did kinetics on inactive mutants of the first phosphorylation site, H554A, and the second phosphorylation site, C384S. A heterodimer consisting of these two mutants was capable of catalyzing both PEP-dependent phosphorylation and mtl/mtl-P exchange (van Weeghel et al., 1991; Weng et al., 1992). *In vivo* studies of Weng et al. (1992) have shown that, when these two phosphorylation site mutants are coexpressed in *E. coli*, growth on mannitol is possible. These complementation studies indicate that phosphoryl group transfer between EII^{mtl} subunits is possible. Phosphoryl group transfer between subunits was also demonstrated for the mannose transporter (Stolz et al., 1993).

The existence of EII^{mtl} dimers and the possibility of phosphoryl group transfer between subunits lead to the question of the role of the individual subunits and the dimer in mannitol phosphorylation and uptake. More specifically, which domains of EII^{mtl} interact and what are the resulting inter- and intramolecular phosphoryl group transfer routes? What is the importance of these subunit interactions for the phosphorylation and transport of mannitol? These questions were studied by *in vivo* and *in vitro* complementation of single and double mutants of EII^{mtl}. EII^{mtl} was mutated at the two phosphorylation sites on the cytoplasmic A and B domains and in the mannitol-binding, membrane-bound C domain, resulting in inactive mutants that show no mannitol binding and/or phosphorylation activity. PEP-dependent mannitol phosphorylation activity, mtl/mtl-P exchange activity, mannitol binding, and mannitol uptake activities were measured for the different mutant enzymes and combinations of enzymes.

MATERIALS AND METHODS

Materials. The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer by Eurosequence bv. Groningen. M13K07 helper phage and the DNA-sequencing kit were obtained from Pharmacia. Klenow enzyme, restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from Boehringer Mannheim. DecylPEG was synthesized by B. Kwant at the Department of Chemistry, University of Groningen. D-[1-^{14}C]Mannitol (2.04 GBq/mmol) was obtained from Amersham. D-[1-^{3}H(N)]Mannitol (976.8 GBq/mmol) was from NEN Research Products, and mannitol 1-phosphate was purchased from Sigma. The GF/F glass microfiber filters were from Whatman. MacConkey agar was obtained from Difco. Enzyme I, enzyme II^{mtl}, HPr, and IIC^{mtl} were purified as described previously (Robillard et al., 1979, 1993; van Dijk et al., 1990; Boer et al., 1994).

Bacterial Strains, Plasmids, and Growth Conditions. The *E. coli* strain CJ236 *dut1, ung1, thi-1, relA1/*pCJ105(cam^r F′) was used to prepare single-stranded template DNA that contains uracil for site-directed mutagenesis (Kunkel et al., 1987). *E. coli* strain JM101 Δ(*lac-proAB*), *supE, thi,* [F′, *traD36, proA*$^+$*B*$^+$, *lacI*^{q}*ZΔM15*] was used for various DNA techniques (Yanish-Peron et al., 1985). The *E. coli* bacterial strain which contains a chromosomal deletion in the wild type *mtlA* gene, LGS322 F$^-$ *thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gatR49, gatA50,* Δ(*mtlA′p*), *mtlD*^{c}, Δ(*gutR′MDBA-recA)*, was used for selection and expression of the mutants of EII^{mtl} (Grisafi et al., 1989).

Plasmid pMamtlA is the expression vector used to produce wild type EII^{mtl} and was also used as the starting vector in the site-directed mutagenesis procedure (van Weeghel et al., 1990). The H554A and C384S mutants of EII^{mtl} were described previously (van Weeghel et al., 1991). Plasmid $pMaIICP_r$ used for the expression of IIC^{mtl} was described previously (Boer et al., 1994). Plasmid pJRD187 was described previously (Davison et al., 1987). Depending on the experiment, strains were grown either on minimal medium plus glucose or on LB medium (10 g of bactotryptone, 5 g of yeast extract, and 10 g of NaCl per liter). Antibiotics were included at concentrations of 25 μg/mL chloramphenicol or 100 μg/mL ampicillin or both, as needed.

Construction of the EII^{mtl} Mutants. The G196D mutation was made following the Kunkel mutagenesis procedure using primer 5′-GAGAAGATATCGTGGTT-3′ and plasmid pMamtlA containing the *mtlA* gene behind the P_{mtl} promoter (Kunkel, 1985). The mutation in plasmid pMaG196D could be detected because a unique *Eco*RV restriction site was created in the *mtlA* gene. The mutated *mtlA* gene was sequenced by the method of Sanger et al. (1977) and was identical to the previously published sequence (Lee & Saier, 1983), except for the mutation introduced. Plasmid pMcG196D, which contains a chloramphenicol resistance gene, was made by replacing the pMa5-8 vector part of plasmid pMaG196D with vector pMc5-8. Plating *E. coli* strain LGS322 containing this mutagenized plasmid on MacConkey agar plates containing 1% D-mannitol gave colonies that had a white, mannitol fermentation-negative, phenotype indicative of a defect in mannitol transport.

Plasmid pMaG196D/C384S was created by replacing the *Sna*BI-*Xba*I fragment of plasmid pMaG196D with the same fragment from plasmid pMaC384S that contains the C384S mutation. In the new plasmid, amino acids 196 and 384 are mutated; this could be checked by cutting the plasmid with *Eco*RV and *Nhe*I, because both mutations created a unique restriction site.

Overexpression of the mutants was achieved by insertion of the λ-P_r promoter with the cI857 λ-repressor gene into the mutant plasmids. For this purpose, an *Eco*RI-*Sal*I fragment containing the P_r promoter and repressor gene was excised from pJRD187 and ligated into the corresponding restriction sites of the plasmid. This strategy for obtaining overexpression was identical to that published previously for the wild type enzyme and IIC^{mtl} (van Weeghel et al., 1990; Boer et al., 1994). The resulting overexpression vector contained the mutant enzyme behind a tandem P_rP_{mtl} promoter.

Plasmid pJRDIIC was constructed for the *in vivo* complementation study. This plasmid contains the same tandem P_rP_{mtl} promoter and IIC^{mtl}-encoding part as the IIC^{mtl} overexpression plasmid $pMaIICP_r$ (Boer et al., 1994), but it has a different origin of replication than the pMa/c5-8 derivatives and can be used in combination with pMcG196D for the

coexpression of IIC^{mtl} and EII^{mtl}-G196D in *E. coli*. A *Bam*HI fragment that encodes for IIC^{mtl} from the overexpression plasmid pMaIICP$_r$ was ligated into plasmid pJRD187, resulting in plasmid pJRDIIC.

Expression, Preparation of Membrane Vesicles, and Purification of the Mutants. *E. coli* strain LGS322 with a *mtlA* gene deletion was used for expression of the mutants of EII^{mtl}. Membrane vesicles were prepared as described previously (Lolkema et al., 1990). Wild type and mutant EII^{mtl} were purified as described (Robillard et al., 1993).

PEP-Dependent Mannitol Phosphorylation and Mannitol/Mannitol Phosphate Exchange Assays. The PEP-dependent phosphorylation kinetics of EII^{mtl} and the mutant proteins were measured in 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, and 0.25% decylPEG at 30 °C. The concentration of enzyme I, HPr, and labeled mannitol depended on the experiment. The samples were incubated for 10 min at 30 °C before the reaction was started with labeled mannitol. Details are given in the figure legends and the text. The volume of the assay mixture was 100 μL. Four 20 μL samples were taken at various times and loaded onto Dowex AG1-X2 columns. A 10 μL sample was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (1987).

Mannitol/mannitol 1-phosphate exchange assays were done at 30 °C in 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 0.25% decylPEG, and a given concentration mannitol 1-phosphate. The exchange reaction was started with [^{3}H]-mannitol after incubation for 10 min at 30 °C. The assay volume and the sample size were the same as for PEP-dependent phosphorylation assays. The assay procedure has been described by Lolkema et al. (1990).

Mannitol Uptake Measurements. For mannitol uptake measurements, cells expressing the EII^{mtl} derivative of interest were grown in the media specified in the text or figure legend until $OD^{600} = 1$, harvested by centrifugation (10 min, 6000*g*), washed with 50 mM KP_i buffer (pH 7.5), and resuspended to an OD^{600} of 10 in the same buffer. For measuring mannitol uptake, 90 μL aliquots of cells with an OD^{600} of 1 were prepared by diluting the cells with 50 mM KP_i buffer (pH 7.5). The uptake measurement was started by adding 10 μL of 35 μM [^{14}C]mannitol to these cells. After the appropriate time, the uptake was quenched by the rapid addition of 2 mL of ice-cold 50 mM KP_i buffer (pH 7.5) containing 1 mM $HgCl_2$. The cell suspension was then rapidly filtered through Whatman GF/F filters, and the vessel and the filter were washed twice with 2 mL of the quenching buffer. The cells on the filters were solubilized with scintillation liquid, and the amount of radioactivity in the cells was determined.

Flow Dialysis. The flow dialysis procedure of Lolkema et al. (1990) was used for measuring the K_d of the enzyme for mannitol. The buffer conditions were 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, and 0.5% decylPEG. The measurements were done at 25 °C, varying the mannitol concentration from 50 to 600 nM for the wild type enzyme.

Protein Determinations. Protein concentrations in the preparations were determined by the method of Lowry (1951) with BSA as the standard. The wild type and mutant EII^{mtl} concentrations were quantitated by flow dialysis as the number of mannitol binding sites extrapolated from a Scatchard plot of mannitol binding or with the pyruvate-burst method (Robillard & Blaauw, 1987). SDS−PAGE was done according to the method of Laemmli (1970).

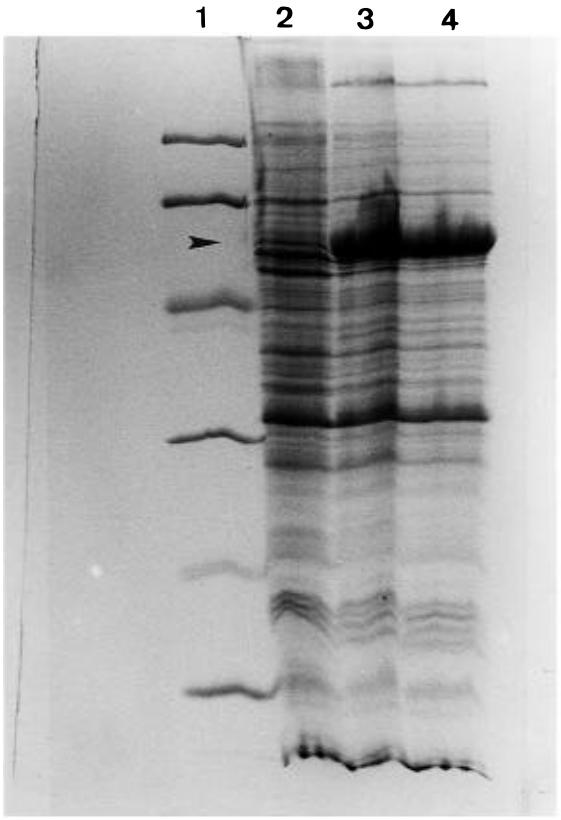

FIGURE 1: SDS−PAGE gel showing the expression of mutant EII^{mtl} G196D/C384S in *E.coli* strain JM101: lane 1, marker proteins of 94, 67, 43, 30, 20.1, and 14.4 kDa; lane 2, membranes of *E. coli* strain JM101 cells that express chromosomally encoded wild type EII^{mtl}; lane 3, membranes derived from *E. coli* strain JM101 cells that overexpress mutant EII^{mtl}-G196D; and lane 4, membranes derived from *E. coli* strain JM101 cells that overexpress double mutant EII^{mtl}-G196D/C384S. The position of EII^{mtl} is indicated with an arrow. All the *E. coli* JM101 cells used for the preparation of these membranes were grown on minimal medium containing glucose to achieve a low expression level of wild type EII^{mtl}.

RESULTS

Characterization of the EII^{mtl}-G196D Mutant. Mutant EII^{mtl}-G196D had a white, mannitol fermentation-negative, phenotype on MacConkey plates with 1% mannitol when expressed in the EII^{mtl} deletion strain LGS322. Membrane vesicles of this strain applied on a SDS−polyacrylamide gel showed a clear band at the EII^{mtl} position, indicating that the mutation had no large effect on the expression of the mutant (Figure 1, lane 3). Apparently, mutation G196D in the C domain results in an enzyme with impaired mannitol binding and/or transport activity. The mutant protein was purified using the same method employed for the wild type EII^{mtl}, and the PEP-dependent mannitol phosphorylation and the mtl/mtl-P exchange kinetics of the purified EII^{mtl}-G196D were examined. PEP-dependent phosphorylation was measured under saturating HPr (17.6 μM) and EI (165 nM) conditions with a mannitol concentration ranging from 1−16 mM in 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, and 0.35% decylPEG. The activity of the mutant was less than 0.1% of the wild type activity at all mannitol concentrations. The same result was found for the mtl/mtl-P exchange kinetics measured in a reaction mixture containing

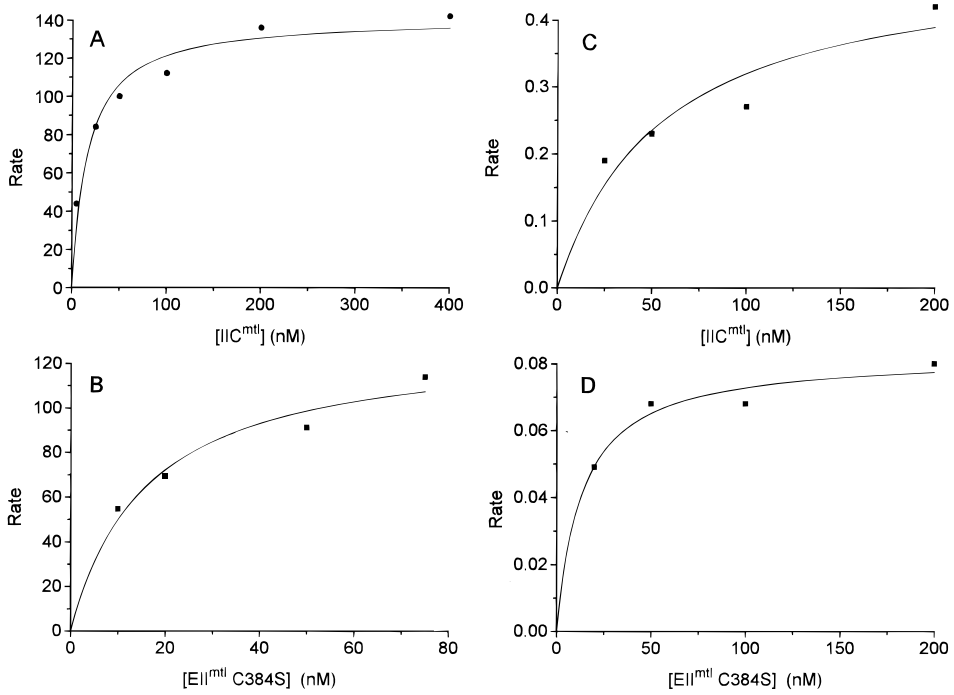

FIGURE 2: Complementation of EII^{mtl}-G196D. (A) Complementation of PEP-dependent phosphorylation activity of 2.9 nM EII^{mtl}-G196D with varying amounts of IIC^{mtl}. (B) Complementation of PEP-dependent phosphorylation activity of 10 nM EII^{mtl}-G196D with varying amounts of EII^{mtl}-C384S. (C) Complementation of mtl/mtl-P exchange activity of 5 nM EII^{mtl}-G196D with varying amounts IIC^{mtl}. (D) Complementation of mtl/mtl-P exchange activity of 20 nM EII^{mtl}-G196D with varying amounts EII^{mtl}-C384S. The assay procedure is identical to what is described in the legend of Table 1. The rates are specific activities in units of nanomoles of mtl-P per minute per nanomole of EII^{mtl}-G196D.

200 nM [^{3}H]mannitol, 250 μM mtl-P, 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, and 0.35% decylPEG. Similar data was also obtained when these measurements were done with membrane vesicles containing EII^{mtl}-G196D.

Since the G196D mutation is situated in the mannitol-binding domain, the lack of activity might reflect a decreased affinity of the enzyme for mannitol. The dissociation constant of purified EII^{mtl}-G196D for mannitol was examined using flow dialysis. In contrast with the situation with the purified wild type enzyme (K_d = 100 nM), no significant mannitol binding could be detected at mannitol concentrations up to 2 μM, indicating that the mutation lowers the affinity for mannitol by at least a factor of 20. The fact that no mannitol phosphorylation activity could be detected up to 16 mM indicates that there is no functional low-affinity binding either. This change in affinity for mannitol makes EII^{mtl}-G196D a suitable candidate for complementation studies to examine the role of C domain interactions in the mechanism of transport and phosphorylation.

Complementation Studies with the EII^{mtl}-G196D Mutant. Previous studies with the EII^{mtl} phosphorylation site mutants, H554A and C384S, have demonstrated that inactive proteins can complement each other, resulting in the restoration of *in vitro* PEP-dependent phosphorylation and mtl/mtl-P exchange activity as well as *in vivo* transport activiy (van Weeghel et al., 1991; Weng et al., 1992). A high-affinity interaction with a K_d in the nanomolar range was also demonstrated to occur at the level of the membrane-bound C domain between purified EII^{mtl} and purified IIC^{mtl}. This experiment and the rates obtained are reproduced schematically from Boer et al. (1994) in panel I of Figure 5. To characterize this interaction further, complementation measurements were performed on the inactive mutant, EII^{mtl}-G196D, and purified IIC^{mtl}. The result of this complementation for the PEP-dependent phosphorylation reaction is shown in Figure 2A. When purified IIC^{mtl} was added to the purified EII^{mtl}-G196D, PEP-dependent phosphorylation activity could be measured. Increasing the IIC^{mtl} concentration led to an increase in phosphorylation activity until saturation was observed. Since EII^{mtl}-G196D can be complemented by an active, high-affinity binding site on IIC^{mtl}, the B domain of EII^{mtl}-G196D must be able to transfer its phosphoryl group to mannitol bound to IIC^{mtl}. There is only one B domain present; IIC^{mtl} lacks a B domain (panel II of Figure 5). To determine whether an additional cytoplasmic domain would have any effect on the above complementation, a similar experiment was done with purified EII^{mtl}-C384S which is inactive because it lacks the second phosphorylation site (panel V of Figure 5). Again, PEP-dependent phosphorylation activity could be measured when EII^{mtl}-G196D was titrated with EII^{mtl}-C384S (Figure 2B). The concentration dependence of EII^{mtl}-C384S was comparable to that observed for IIC^{mtl} as was the maximal activity. Panels C and D of Figure 2 show the result for the mtl/mtl-P exchange reaction. Both IIC^{mtl} and EII^{mtl}-C384S can complement EII ^{mtl}-G196D in this reaction and show an enzyme concentration dependence similar to the phosphorylation kinetics. As usual, the specific activity of the exchange reaction is only a fraction of the phosphorylation activity (Table 1). These results show that active forms of EII^{mtl} capable of *in vitro* PEP-dependent phosphorylation and mtl/mtl-P exchange can be created from mutants that, by themselves, are inactive due to nonfunctional B or C domains. However, a quantitative difference is evident when comparing the rates in panel II and V *versus* that in panel I; complementation with active enzyme results in much higher rates. This will be treated in the Discussion.

In order to determine whether *in vivo* mannitol transport could be restored and not just *in vitro* phosphorylation, a

Table 1: Complementation of EII^{mtl}

EII^{mtl} species	PEP-dependent phosphorylation[b] [nmol of mtl-P min^{-1} (nmol of EII^{mtl})$^{-1}$]	mtl/mtl-P exchange[d] [nmol of mtl-P min^{-1} (nmol of EII^{mtl})$^{-1}$]
G196D	nd	nd
C384S	nd	nd
IIC^{mtl}	nd	nd
G196D/C384S	nd	nd
G196D + IIC^{mtl} [c]	130	0.4
G196D + C384S	125	0.1
EII^{mtl} + IIC[a]	928	5.1
EII^{mtl} + C384S	1049	7
EII^{mtl} + G196D	900	6.3
EII^{mtl} + G196D/C384S	990	4.6

[a] The data in rows 7−10 are specific activities for the wild type enzyme (EII^{mtl}) at saturating concentrations of the second component. [b] The PEP-dependent phosphorylation was measured at 30 °C with a 100 μL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decylPEG, 60 μM [^{3}H]mannitol, 165 nM EI, 17.6 μM HPr, and the purified EII^{mtl} listed in the table. Four 20 μL aliquots were taken at various times and loaded onto Dowex AG1-X2 columns. A 10 μL aliquot was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (1987). nd indicates not detectable and means that the phosphorylation activity was less than 1 nmol of mtl-P min^{-1} (nmol of EII^{mtl})$^{-1}$. [c] The data in rows 5 and 6 are the specific activities for the mutant enzyme (G196D) in the presence of saturating concentrations of the second component. [d] The mannitol/mannitol-P exchange was measured at 30 °C with a 100 μL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 0.25% decylPEG, 250 μM mannitol 1-phosphate, 200 nM [^{3}H]mannitol, and membrane vesicles containing the mutant. The further assay procedure was identical to that described above. nd indicates not detectable and means that the exchange activity was less than 0.01 nmol of mtl-P min^{-1} (nmol of EII^{mtl})$^{-1}$.

two-plasmid expression system was developed to allow the coexpression of EII^{mtl} derivatives in *E. coli*. Strain LGS322 was transformed either with plasmid pJRDIIC expressing IIC^{mtl} or with pMcG196D expressing EII^{mtl}-G196D; it was also transformed with the two plasmids together. Cells grown at 37 °C expressing IIC^{mtl} or EII^{mtl}-G196D had a white phenotype on MacConkey plates containing 1% mannitol, while cells containing the two plasmids had a red/pink phenotype, indicative of mannitol fermentation. The uptake of mannitol was then measured in cells that expressed IIC^{mtl} , EII^{mtl}-G196D, or both proteins simultaneously. Figure 3 shows the initial uptake of 3.5 μM [^{3}H]mannitol in whole cells. The uptake levels of mannitol in the cells expressing IIC^{mtl} (■) and EII^{mtl}-G196D (◆) are the same as that found for the control cells that lacked the plasmid (▲); virtually no uptake of mannitol occurred. Coexpression of the two proteins led to a significant increase in the initial rate and level of mannitol uptake (●). Clearly, EII^{mtl}-G196D can also be complemented by IIC^{mtl} in the native membrane, resulting in an enzyme that is capable of mannitol transport.

Taken together, these results and all previously published data show that EII^{mtl}, inactivated by a mutation in any one of the three domains, can be reclaimed by complementing it with a partner in which the complementary domain is active. For this to be possible, phosphoryl group transfer from one EII^{mtl} subunit to another must occur; furthermore, it is sufficient to catalyze *in vitro* phosphorylation and exchange as well as *in vivo* transport. The mere observation of phosphoryl group transfer between EII^{mtl} subunits, however, does not prove that this is the preferred route in wild type EII^{mtl}. It is equally possible that a phosphoryl group is passed

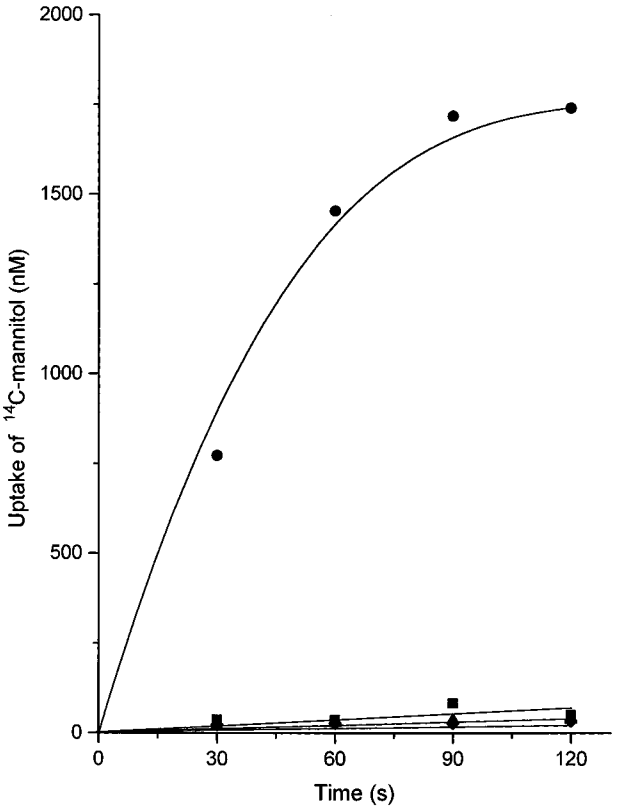

FIGURE 3: Mannitol uptake activity in *E. coli*. (▲) *E. coli* LGS322 without a plasmid (0.27 mg/mL protein). (■) *E. coli* LGS322 containing plasmid pJRDIIC (0.18 mg/mL protein). (◆) *E. coli* LGS322 containing plasmid pMcG196D (0.12 mg/mL protein). (●) *E. coli* LGS322 containing plasmids pJRDIIC and pMcG196D (0.23 mg/mL protein). Aliquots (90 μL) of cells with an OD^{600} of 1 were prepared by diluting the cells with 50 mM KP_i buffer (pH 7.5). The uptake measurement was started by adding 10 μL of 35 μM [^{14}C]mannitol to these cells. After the appropriate time, the uptake was quenched by the rapid addition of 2 mL of ice-cold 50 mM KP_i buffer (pH 7.5) containing 1 mM $HgCl_2$. The cell suspension was than rapidly filtered across Whatman GF/F filters, and the vessel and filter were washed twice with 2 mL of the quenching buffer. The cells on the filters were solubilized with scintillation liquid, and the amount of radioactivity in the cells was determined.

from the A domain to the B domain and then to mannitol on the C domain of a single EII^{mtl} subunit. The following section addresses this issue for both the *in vitro* and *in vivo* processes using a double mutant.

Complementation of the EII^{mtl}-G196D/C384S Mutant with EII ^{mtl}-H554A and Wild Type EII^{mtl}. EII^{mtl} -G196D/C384S possesses both an inactive C and B domain. To determine whether the A domain was still functional, it was complemented with EII^{mtl}-H554A, a protein with an inactive A domain but active B and C domains. The PEP-dependent phosphorylation activity could be restored in a saturatable manner when increasing concentrations of EII^{mtl}-G196D/C384S were combined with EII^{mtl}-H554A just as shown in Figure 2 for the other mutants and domains. The occurrence of saturatable activity in the low nanomolar enzyme concentration range is evidence that the double mutant is still able to form functional heterodimers.

Complementation of the double mutant with wild type EII^{mtl} enables us to address the issue of the role of the second subunit of the dimer in the phosphorylation and transport mechanism. If phosphoryl transfer across the dimer interface from the B domain on one subunit to the mannitol−C domain complex on the other subunit is an essential element of the

phosphorylation and transport mechanism, then heterodimers consisting of one wild type subunit and one BC domain double mutant should be inactive (panel VI of Figure 5). The experiments were carried out exactly as those in Figure 2 with a fixed concentration of wild type enzyme and increasing concentrations of the double mutant. The results of these experiments are given in Table 1 and panel VI of Figure 5. Addition of the double mutant to the wild type enzyme does not lead to a decrease in PEP-dependent phosphorylation or mtl/mtl-P exchange activity but, rather, to an increase just as seen by Boer et al. (1994) upon addition of IIC^{mtl} to wild type enzyme. These high rates indicate that, although phosphoryl group transfer across the subunit interface is possible, the preferred route lies within a single subunit. The question remains as to whether this is also true for mannitol transport. This was addressed by the coexpression of wild type EII^{mtl} and EII^{mtl}-G196D or EII^{mtl}-G196D/C384S.

E. coli strain JM101 which expresses wild type EII^{mtl} was grown on minimal medium containing glucose instead of mannitol to achieve a low expression level of wild type EII^{mtl}. Low wild type expression levels were chosen for two reasons. (1) Good initial uptake rates could be measured, and (2) the expression level of the wild type enzyme would be much lower than that of the mutant EII^{mtl}, resulting in a high percentage of heterodimers containing one wild type subunit and one mutant subunit. That linear initial rates of mannitol uptake can be measured in cells grown on glucose is clearly seen in Figure 4 (■). SDS−polyacrylamide gel electrophoresis (Figure 1, lane 2) also shows that only low amounts of EII^{mtl} are present in these cells. The same strain was transformed with plasmid pMaG196D or pMaG196D/C384S and grown under the same conditions until the cells reached an OD^{600} of 0.6, and then expression of plasmid-encoded protein was initiated by thermoinduction. The SDS−polyacrylamide gel in Figure 1 shows that the level of chromosomally expressed wild-type enzyme (lane 2) is much lower than that of EII^{mtl}-G196D or EII^{mtl}-G196D/C384S derived from the overexpression plasmids (lanes 3 and 4, respectively). The initial uptake rates of the cells that overexpressed the mutant G196D or G196D/C384S, however, are similar to that of cells that only expressed wild type EII^{mtl} (Figure 4). The presence of an inactive mutant subunit does not affect the rate of uptake of mannitol, leading to the conclusion that mannitol uptake by the dimer occurs via a single subunit, without the necessary involvement of the other subunit of the dimer.

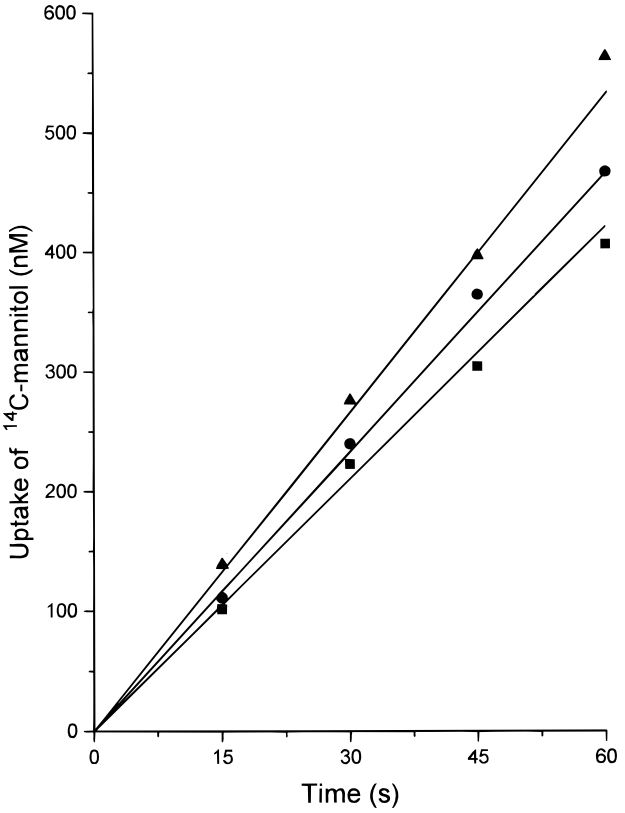

FIGURE 4: Uptake measurements with EII^{mtl}-G196D/C384S and wild type EII^{mtl}. (■) *E. coli* JM101 cells that express chromosomally encoded wild type EII^{mtl} (0.26 mg/mL protein). (●) *E. coli* JM101 cells that overexpress mutant EII^{mtl}-G196D (0.29 mg/mL protein). (▲) *E. coli* JM101 cells that overexpress double mutant EII^{mtl}-G196D/C384S (0.33 mg/mL protein). All the cells in these uptake measurements were grown on minimal medium containing glucose to achieve a low expression level of wild type EII^{mtl}. The uptake of radioactive mannitol was measured as described in the legend of Figure 3.

DISCUSSION

Phosphoryl Group Transfer by EII^{mtl}. The various heterodimer experiments presented above are summarized in Figure 5. We started with the observation, made previously by Boer et al. (1994), that the addition of purified IIC^{mtl} to purified EII^{mtl} (panel I) resulted in a stimulation of the specific phosphorylation and exchange activity with an apparent affinity of IIC^{mtl} for EII^{mtl} of less than 10 nM. This observation can be interpreted in two ways: (i) that heterodimers are being formed or (ii) that addition of increasing concentrations of IIC^{mtl} is shifting the equilibrium of a monomeric population of intact EII^{mtl} back into the homodimeric form. The experiment in panel II shows that the heterodimer explanation is the correct one. If IIC^{mtl} was only shifting a monomeric population of intact EII^{mtl} back to the homodimer form, no activity would be observed in this panel since the intact enzyme is mutated in the C domain and is inactive. The experiment in panel I does not however discriminate between phosphoryl group transfer from the B to the C domain within one subunit *versus* transfer across the subunit interface because both subunits in the heterodimer possess active C domains. However, when IIC^{mtl} was added to EII^{mtl}-G196D, the PEP-dependent phosphorylation and mtl/mtl-P exchange activities of these inactive species were restored (panel II). The saturation kinetics that were observed are indicative of the formation of a complex between IIC^{mtl} and EII^{mtl}-G196D where the phosphoryl group must transfer across the subunit interface from the active B domain on EII^{mtl}-G196D to the mannitol bound to IIC^{mtl}. In this case, however, the rates of both phosphorylation and exchange are approximately 10-fold lower than in panel I. The lower rates are not due to the inactive nature of the C domain of EII^{mtl}-G196D, as such, because high rates are observed with a heterodimer of wild type EII^{mtl} and EII^{mtl}-G196D (panel III). Consequently, the lower rates in panel II must be due to the necessity of the phosphoryl group to transfer across the subunit interface.

The experiment in panel V indicates that the presence of the inactive B domain does not prevent such a transfer, but again, the rates are approximately 10-fold lower than when a wild type subunit is present. Here as well, the inactive nature of the B domain is not responsible for the lower rates

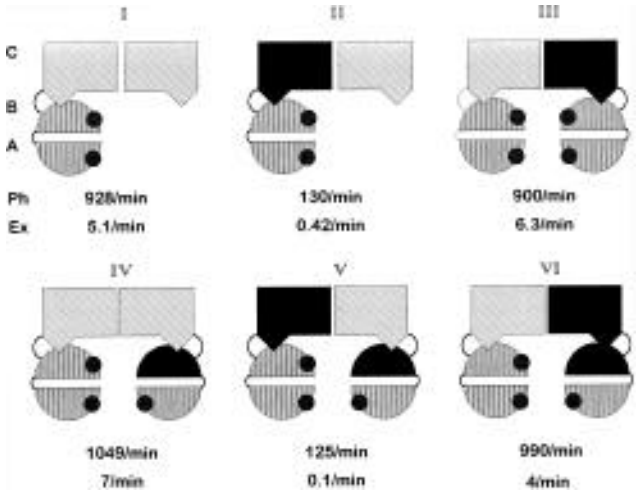

FIGURE 5: Schematic representation of the complementation experiments. The panels represent the EII^{mtl} heterodimers that have been studied. Inactive domains are represented in black, and phosphorylation sites are indicated with a circle. Ph = PEP-dependent phosphorylation activity, and Ex = mtl/mtl-P exchange activity. (I) EII^{mtl} and IIC^{mtl}, (II) G196D and IIC^{mtl}, (III) EII^{mtl} and G196D, (IV) EII^{mtl} and C384S, (V) G196D and C384S, and (VI) EII^{mtl} and G196D/C384S. The rates are specific activities expressed as nanomoles per minute per nanomole of wild type EII^{mtl} in panels I, III, IV, and VI and nanomoles per minute per nanomole of EII^{mtl}-G196D in panels II and V at saturating levels of the other component.

because, when a wild type subunit is present (panel IV), high rates are observed. Finally, complementation of the double mutant EII^{mtl}-G196D/C384S with mutant EII^{mtl}-H554A demonstrates that the double mutant is capable of forming heterodimers. When it forms a heterodimer with wild type EII^{mtl} (panel VI), high rates of phosphorylation and exchange are observed.

From this collection of data, we conclude that the preferred phosphorylation route between the B and C domains of the EII^{mtl} dimer lies within a single subunit; phosphoryl group transfer across the subunit interface can occur but only at a much slower rate. Weng et al. (1992) and Weng and Jacobson (1993) reported *in vivo* mutant complementation data using pairs of A, B, and C domain mutants expressed in *E. coli*. A and B domain mutants were capable of complementing each other as were A and C domain mutants when *in vivo* mannitol fermentation and *in vitro* phosphorylation in permeabilized cells were monitored. However, B and C domain mutants could not complement each other in *in vivo* fermentation or *in vitro* phosphorylation. This led Weng and Jacobson to conclude that high rates of phosphorylation required an active B and C domain on the same subunit. The data presented in this report support this conclusion. Even though Table 1 and Figure 5 report substantial levels of activity in the B and C domain complementation experiments, we must emphasize that these rates are achieved by using large excesses of one mutant relative to the other. In their *in vivo* complementation experiments, Weng and Jacobson (1993) would have had comparable concentrations of both mutant enzymes leading to a large fraction of inactive homodimers and, consequently, much lower activities that we have reported here. This is most probably the reason for the inability of their cells to carry out mannitol fermentation, even though we are able to measure "significant" levels of phosphorylation activity *in vitro*.

Mannitol Transport by EII^{mtl}. Coexpression of IIC^{mtl} and EII^{mtl}-G196D in *E. coli* indicates that these two enzymes also associate in their natural environment to form a minimal configuration in the membrane that is able to catalyze mannitol transport. When the double mutant, EII^{mtl}-G196D/C384S, is overexpressed relative to the uninduced wild type EII^{mtl} in *E. coli*, heterodimers will also form and, due to the large excess of double mutant relative to wild type enzyme, we expect virtually all wild type EII^{mtl} to be present in the heterodimer form with one active and one completely inactive subunit. Since the initial uptake rates are very similar in the presence and absence of the double mutant, we must conclude that a single subunit in the dimer is capable of catalyzing the whole transport reaction as well as the phosphorylation reaction. No negative dominance is observed.

The *in vivo* and *in vitro* complementation experiments show that EII^{mtl} is a prime example of a protein in which facultative domain swapping occurs (Bennett et al., 1995), but there is a clear preference for the domains to function with their partners in the same subunit. If a single subunit is sufficient, even optimal, for catalyzing both phosphorylation and transport, what is the rationale for the EII^{mtl} dimer? Two reasons which could apply to transport proteins in general are as follows.

(i) Subunit interactions may result in pore formation. The monomeric subunits of aquaporin form a tetramer with a central cavity, and within this cavity, each monomer forms a narrow pore (Engel et al., 1994). As in the case of EII^{mtl}, Lindenthal and Schubert (1991) demonstrated that a monomeric form of the erythrocyte transporter transports anions but electron diffraction showed a dimer with one central cavity (Wang et al., 1993). The dimer interface of EII^{mtl} may also form a pore which is involved in providing the transport route for the sugar across the membrane. Robillard and Beechey (1986), using membrane impermeable reagents, provided clear evidence for the occurrence of such a pore in the *E. coli* EII^{glc} which, like EII^{mtl}, is also known to be a dimer (Erni, 1986).

(ii) Cooperative binding site interactions may facilitate rapid transport. The erythrocyte glucose transporter is a tetramer with cooperative binding site interactions between the subunits. In this protein, subunit association is important for rapid substrate translocation (Zottola et al., 1995; Coderre et al., 1995). Pas et al. (1988) presented evidence for one high-affinity and one low-affinity mannitol binding site per dimer, implying cooperative subunit interactions which Lolkema et al. (1993) also suggested could be involved in rapid substrate translocation.

More mechanistic and structural work will be necessary to resolve this issue.

REFERENCES

Bennett, M. J., Schlunegger, M. P., & Eisenberg, D. (1995) *Protein Sci. 4*, 2455−2468.

Boer, H., ten Hoeve-Duurkens, R. H., Schuurman-Wolters, G. K., Dijkstra, A., & Robillard, G. T. (1994) *J. Biol. Chem. 269*, 17863−17871.

Buhr, A., Daniels, G. A., & Erni, B. (1992) *J. Biol. Chem. 267*, 3847−3851.

Coderre, P. E., Cloherty, E. K., Zottola, R. J., & Carruthers, A. (1995) *Biochemisty 34*, 9762−9773.

Davison, J., Heusterspreute, M., Chevalier, N., & Brunel, F. (1987) *Gene 60*, 227−235.

Engel, A., Walz, T., & Agre, P. (1994) *Curr. Opin. Struct. Biol. 4*, 545−553.
Erni, B. (1986) *Biochemistry 25*, 305−312.
Grisafi, P. L., Scholle, A., Sugiyama, J., Briggs, C., Jacobson, G. R., & Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719−2727.
Khandekar, S. S., & Jacobson, G. R. (1989) *J. Cell. Biochem. 39*, 207−216.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−383.
Laemmli, U. K. (1970) *Nature 277*, 680−685.
Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 285*, 10761−10767.
Leonard, J. F., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10757−10760.
Lindenthal, S., & Schubert, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6540−6544.
Lolkema, J. S., & Robillard, G. T. (1990) *Biochemistry 29*, 10120−10125.
Lolkema, J. S., & Robillard, G. T. (1992) *New Compr. Biochem. 21*, 135−168.
Lolkema, J. S., Swaving Dijkstra, D., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1990) *Biochemistry 29*, 10659−10663.
Lolkema, J. S., Kuiper, H., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1993) *Biochemistry 32*, 1396−1400.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Manayan, R., Tenn, G., Yee, H. B., Desai, J. D., Yamada, M., & Saier, M. H., Jr. (1988) *J. Bacteriol. 170*, 1290−1296.
Pas, H. H., Ellory, J. C., & Robillard, G. T. (1987) *Biochemistry 26*, 6689−6696.
Pas, H. H., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5520−5525.
Postma, P. W., Lengeler, J. W., & Jacobson, G. R. (1993) *Microbiol. Rev. 57*, 543−574.
Robillard, G. T., & Beechey, R. B. (1986) *Biochemistry 25*, 1346−1354.
Robillard, G. T., & Blaauw, M. (1987) *Biochemistry 26*, 5796−5803.
Robillard, G. T., Dooijewaard, G., & Lolkema, J. S. (1979) *Biochemistry 18*, 2984−2989.
Robillard, G. T., Boer, H., van Weeghel, R. P., Wolters, G., & Dijkstra, A. (1993) *Biochemistry 32*, 9553−9562.
Roossien, F. F., & Robillard, G. T. (1984a) *Biochemistry 23*, 5682−5685.
Roossien, F. F., & Robillard, G. T. (1984b) *Biochemistry 23*, 211−215.
Roossien, F. F., Blaauw, M., & Robillard, G. T. (1984) *Biochemistry 23*, 4934−4939.
Roossien, F. F., van Es-Spiekman, W., & Robillard, G. T. (1986) *FEBS Lett. 196*, 284−290.
Ruiter, G. J. G., van Meurs, G., Verwey, M. A., Postma, P. W., & van Dam, K. (1992) *J. Bacteriol. 174*, 2843−2850.
Saier, M. H., Jr. (1980) *J. Supramol. Struct. 14*, 281−294.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 4046−4051.
Stolz, B., Huber, M., Markovic-Housley, Z., & Erni, B. (1993) *J. Biol. Chem. 268*, 27094−27099.
Sugiyama, J. E., Mahmoodian, S., & Jacobson, G. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9603−9607.
van Dijk, A. A., de Lange, L. C. M., Bachovchin, W. W., & Robillard, G. T. (1990) *Biochemistry 29*, 8164−8171.
van Weeghel, R. P., Keck, W., & Robillard, G. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2613−2617.
van Weeghel, R. P., van der Hoek, Y. Y., Pas, H. H., Elferink, M., Keck, W., & Robillard, G. T. (1991) *Biochemistry 30*, 1768−1773.
Wang, D. N., Kühlbrandt, W., Sarabia, V. E., & Reithmeier, R. A. F. (1993) *EMBO J. 12*, 2233−2239.
Weng, Q. P., & Jacobson, G. R. (1993) *Biochemistry 32*, 11211−11216.
Weng, Q. P., Elder, J., & Jacobson, G. R. (1992) *J. Biol. Chem. 267*, 19529−19535.
Yanish-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103−109.
Zottola, R. J., Cloherty, E. K., Coderre, P. E., Hansen, A., Hebert, N. D., & Carruthers, A. (1995) *Biochemistry 34*, 9734−9747.

BI9611016